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THE DEGRADATION OF
PYRIMIDINES AND PURINES
FOR TRACER WORK

Diss. med.
Stockholm. Karolinska Institutet
22 APR. 1953

STOCKHOLM 1953



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From the Biochemical Department, Karolinska Institutet

STOCKHOLM, SWEDEN

THE DEGRADATION OF
PYRIMIDINES AND PURINES
FOR TRACER WORK

by

ULF LAGERKVIST

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*This treatise is a summary of the following
three publications:*

- I. The Isolation of Nitrogen 1 and 3 as Methylamine and Ammonia from Pyrimidine Ribosides. Acta Chem. Scand. 4, 543 (1950).
- II. The Degradation of Pyrimidines for Tracer Work. Acta Chem. Scand. 7, 114 (1953).
- III. The Incorporation of Ammonia into Uric Acid in Pigeons and Ribonucleic Acid Pyrimidines in Rats. Arkiv för kemi Bd 5, Nr 54, 569.



Almqvist & Wiksell

BOKTRYCKERI AKTIEBOLAG

UPPSALA 1953

INTRODUCTION

During the last decade isotopic nitrogen and carbon have been extensively used to investigate the synthesis of biologically important substances both in vivo and in vitro. In such investigations it is often necessary to be able to determine the distribution of isotope within the molecule whose synthesis is being investigated. Detailed informations regarding the isotope excess of the individual atoms in a molecule that has incorporated isotope from a labeled precursor will in many cases enable us to decide whether or not the precursor in question is directly related to the synthesis of this molecule.

Investigations of this kind call for suitable degradation methods that permit the isolation of the individual nitrogen and carbon atoms without contamination with the other atoms in the molecule or with nitrogen or carbon from the reagents used in the degradation procedure. A contamination of the nitrogen fractions with carbon and vice versa is, of course, quite harmless. The use of such degradation methods for the localization of isotope in tracer experiments has thrown light on many problems in intermediary metabolism. It will suffice to mention the basic work of BUCHANAN *et al.* (1) on the precursors of uric acid carbon and the ingenious investigations of porphyrine metabolism recently published by SHEMIN *et al.* (2, 3).

The chief obstacle in the elaboration of degradation methods for use in tracer work is the usually minute quantities available for degradation. In many cases it is impossible to use conventional methods for isolation and purification such as distillation and crystallization and many of the reactions used in ordinary organic chemistry can be applied only with great difficulty on the semi-micro scale. The introduction of partition chromatography by Martin and co-workers and the extensive use of chromatography on ion exchange resins in recent years has been of the greatest value for the elaboration of degradation methods. It would be safe to say that the degradation methods reported later on in this treatise could not have been worked out, at least not on the semi-micro scale, without the use of chromatographic separations.

In some cases the origin of the fractions obtained in the final steps of the degradation is quite obvious from the chemical operations used but in most cases it is necessary to establish the reliability of the method in some way. This is often done by synthesizing the substance to be degraded labeled with isotope in suitable positions and then degrading it with the method to be tested. It is of course also possible to investigate the accuracy of a degradation without using isotopes or by using a combination of isotopes and other methods such as an analysis of the chromatographic behaviour of a given fraction compared to possible contaminating substances. An example of such considerations can be seen in paper III.

In spite of the great interest in pyrimidine and purine metabolism that has arisen in recent years, suitable degradation methods for the pyrimidines and for all the nitrogen atoms of the purines have been lacking. BUCHANAN *et al.* (1) were able to separate the nitrogen atoms of the pyrimidine and imidazole moieties of uric acid from each other and several authors have used the isolation of nitrogen atom 7 after acid hydrolysis of purines in tracer studies (4, 5). No attempts, however, have been made to separate nitrogen atoms 1 and 3.

In paper I a method for the separation of nitrogen atoms 1 and 3 in uridine is described. In a preliminary communication (6) a degradation of uracil was reported in which carbon atom 2 was obtained as urea while the oxalic acid formed at the same time was believed to be representative of carbon atoms 5 and 6. A similar degradation was published at the same time by HEINRICH and WILSON (7). By degradation of labeled uracil it was soon realized, however, that the oxalic acid was not representative of carbon atoms 5 and 6 and therefore a new degradation method was worked out that permitted the isolation of every carbon atom in the uracil molecule (paper II). Finally, a method for the separation of nitrogen atoms 1 and 3 in the purines has been elaborated and using these methods the distribution of isotope from N¹⁵-labeled ammonia and aspartic acid in ribonucleic acid pyrimidines in rats and uric acid in pigeons has been investigated (paper III).

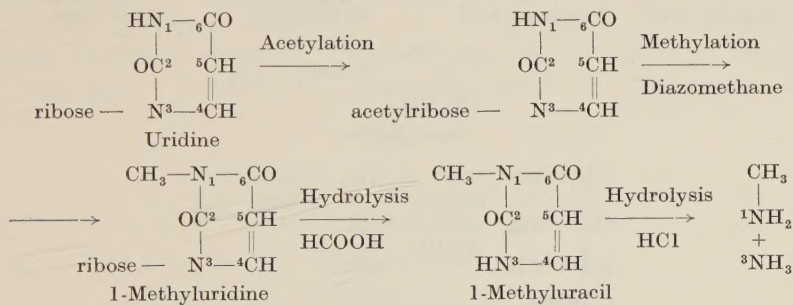
METHODS

The Separation of the Pyrimidine Nitrogen Atoms

PAPER I

The method was worked out for uridine but it can also be applied to cytidine after deamination of this substance to uridine (8). A general outline of the degradation is given below together with a discussion of the reliability of the method.

About 20 mg uridine (0.08 mM) were dissolved in acetone-water and acetylated with ketene from a ketene lamp. After evaporation to dryness in vacuo the residue was dissolved in methanol-acetone and treated with an excess of diazomethane in ether (9). The remaining diazomethane was destroyed after three hours with glacial acetic acid and the sample hydrolyzed with formic acid according to VISCHER and CHARGAFF (10). The resulting 1-methyluracil was purified by chromatography on starch and then degraded to methylamine and ammonia by oxidation with KMnO_4 and subsequent hydrolysis with strong alkali. In a modification of this method, published in paper III, the methyluracil was converted to methylamine and ammonia by hydrolysis with HCl in a bombtube. Methylamine and ammonia were separated by chromatography on starch using the technique described by MOORE and STEIN (11, 12) for the separation of amino acids. The over all yield was about 70 % using the modification compared to 45-50 % with the old method.



It was not considered necessary to make any special tests of the reliability of the method. The treatment of uridine with diazomethane could only result in a methylation of one of the two nitrogen atoms and because the ribose is known to be attached to nitrogen atom 3 the methylation must occur in position 1. Since there is no reason to believe that the following degradation should bring about an exchange of methyl groups between nitrogen atoms 1 and 3 the methylamine should consequently be representative of nitrogen atom 1 and the ammonia of nitrogen atom 3.

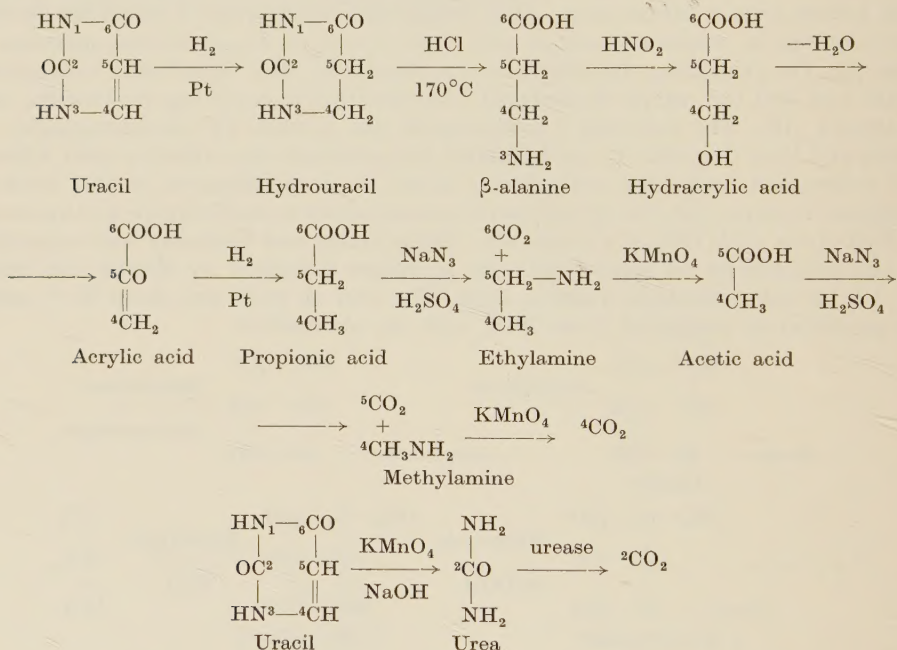
The Separation of the Pyrimidine Carbon Atoms

PAPER II

The degradation was worked out for uracil but it can also be applied to cytosine after deamination (8) of this substance to uracil. A general survey of the method will be given here together with a discussion of its accuracy.

For the separation of carbon atoms 4, 5 and 6, uracil (0.8–1 mM) was dissolved in a 0.5 % water solution of gum arabic and hydrogenated under pressure with chloroplatinic acid as catalyst (13). The resulting hydrouracil was hydrolyzed to β -alanine (14) with HCl and the β -alanine that represented carbon atoms 4, 5 and 6 isolated by chromatography on Dowex 50. Deamination of this compound to hydroacrylic acid and subsequent dehydration yielded acrylic acid (15) and on catalytic hydrogenation propionic acid was obtained. The propionic acid was isolated by chromatography on celite with chloroform-butanol according to MOSBACH *et al.* (16) and then degraded by two Schmidt decarboxylations according to PHARES (17).

Carbon atom 2 was isolated as urea after oxidation of uracil (0.10–0.15 mM) with KMnO_4 and hydrolysis with alkali. Carbon atom 2 was liberated from the urea as CO_2 after treatment with urease.



In order to test the reliability of the degradation uracil labeled with C^{13} in position 5 and C^{14} in position 6 was synthesized from 3- C^{13} , 4- C^{14} -aspartic acid.¹ The aspartic acid was deaminated to malic acid and on condensation with urea according to DAVIDSON and BAUDISCH (18) uracil was obtained in 25 % yield calculated on aspartic acid. Degradation of the labeled uracil showed that the carboxyl and methylene

¹ I am greatly indebted to Dr. GÖSTA EHRENSVÄRD for the generous gift of this substance.

carbons of propionic acid were representative of carbon atoms 6 and 5 respectively in the uracil molecule. The methyl carbon must consequently be representative of carbon atom 4 since the degradation of labeled uracil clearly showed that carbon atoms 5 and 6 did not enter this position in propionic acid. It is of course quite obvious that carbon atom 2 could not give rise to the methyl group of propionic acid.

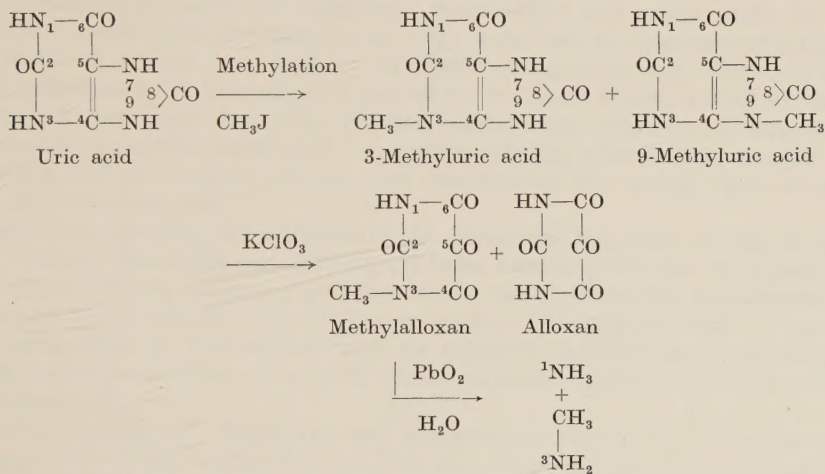
It was not considered necessary to investigate the origin of the urea obtained after oxidation and alkaline hydrolysis since it is obvious from the structure of the uracil molecule that carbon atom 2 is the only one that could give rise to urea.

The Separation of the Purine Nitrogen Atoms

PAPER III

The method was worked out for uric acid, but can also be applied to adenine, guanine, hypoxanthine and xanthine since these substances are easily converted to uric acid by deamination (adenine and guanine) and oxidation with xanthine oxidase. A summary of the method and a discussion of its validity is given below.

Uric acid (0.3–0.5 mM) was methylated with methyl iodide in alkaline solution to a mixture of 70 % 3-methyluric acid and 30 % 9-methyluric acid (19). 3- and 9-methyluric acid crystallize in a mixed crystal and were therefore obtained together after chromatography on starch and crystallization from water. The methyluric acid was oxidized with KClO_3 to methylalloxan and alloxan (20) and these substances separated by chromatography on starch. The methylalloxan was further degraded to methylamine and ammonia by oxidation with PbO_2 (21) and hydrolysis with dilute HCl . Methylamine and ammonia were isolated as described in paper I. Nitrogen atom 7 was obtained as glycine after hydrolysis of uric acid with HCl (22) and isolated by chromatography on Dowex-50 with N HCl according to STEIN and MOORE (23).



The origin of glycine from nitrogen atom 7 has been established beyond doubt (24) but it still remained to be shown that nitrogen atoms 1 and 3 were separated from each other and from nitrogen atoms 7 and 9 under the conditions

described above. For this purpose a synthesis of 7,9- N^{15} -uric acid from N^{15} -urea (25) was undertaken using the well known method of BEHREND and ROOSEN (26) with some modifications. Degradation of the labeled uric acid showed that nitrogen atoms 1 and 3 were almost completely separated from nitrogen atoms 7 and 9, the contamination with nitrogen from these positions being 4 % or less. In spite of this contamination the separation was considered good enough for practical purposes.

Regarding the separation of nitrogen atoms 1 and 3 from each other it could be demonstrated that the methyluric acid fraction obtained by chromatography after methylation of uric acid could be separated from 1-methyluric acid and 1,9-dimethyluric acid under the chromatographic conditions used. It was furthermore demonstrated that this fraction did not contain any methyl derivatives of uric acid with methyl groups in position 7. Methyl derivatives with methyl groups in both positions 1 and 3, on the other hand, would give rise to dimethylalloxan that can be separated from methylalloxan as can be seen in Fig. 3 in paper III. No peak corresponding to dimethylalloxan was obtained after oxidation with $KClO_3$. The methyluric acid fraction had the same R -value as a mixture of 3-methyluric acid and 9-methyluric acid (ζ -methyluric acid) (27) when subjected to chromatography on starch using either propanol-HCl or butanol-water as eluting agents. It also showed the same light absorption curve determined in N HCl using the Beckman spectrophotometer.

Under such circumstances it seems justified to regard the ammonia and methylamine obtained in the last step of the degradation as representative of nitrogen atoms 1 and 3 respectively. This is furthermore supported by the fact that in the experiment with N^{15} -ammonia on pigeons reported in paper III the isotope excess of the methylamine was 8 times that of the ammonia. Such a result could not have been obtained if the degradation had brought about a more extensive randomization of nitrogen atoms 1 and 3.

EXPERIMENTS WITH N^{15} -LABELED AMMONIA AND ASPARTIC ACID

PAPER III

Two types of nucleic acids are distinguished: desoxyribose nucleic acid (DNA) which is located in the cell nucleus and ribose nucleic acid (RNA) which is found mainly in the cytoplasm but to a lesser degree also in the nucleus. These two types differ not only regarding their carbohydrate constituents but also with respect to their pyrimidine bases. DNA contains the pyrimidine bases thymine and cytosine while uracil and cytosine are found in RNA. As to the purines, adenine and guanine appear in both DNA and RNA.

In recent years there has been an increasing interest in the metabolism of the pyrimidines. However, the investigations of possible precursors have mainly dealt with the ability of substances with preformed pyrimidine rings to function as precursors. The free pyrimidines uracil, cytosine and thymine were not incorporated into nucleic acid pyrimidines when administered to rats (28, 29) while the corresponding ribosides and desoxyribosides were found to be utilized (30, 31). The only free pyrimidine so far found to be more widely used as a precursor in pyrimidine synthesis is orotic acid. This substance, first discovered in milk (32), promoted growth in several microorganisms that require pyrimidines for full growth (33, 34, 35). In 1947 MITCHELL and HOULAHAN (36) proposed the theory that pyrimidine synthesis proceeds via oxalacetic, fumaramino and orotic acids. By using N^{15} -labeled orotic acid ARVIDSON et al. (37) were able to demonstrate a high incorporation of isotope into nucleic acid pyrimidines in the rat. That orotic acid may well be a normal intermediate in pyrimidine synthesis is born out by REICHARDS experiments in rat liver slices (38). When N^{15} -ammonium chloride was administered together with unlabeled orotic acid a very high labeling of orotic acid resulted while the nucleic acid pyrimidines incorporated considerably less isotope than in experiments without orotic acid.

Information regarding the synthesis of the pyrimidine ring from smaller molecules is very limited. HEINRICH and WILSON (7) have demonstrated the incorporation of isotope from $C^{14}O_2$ in position 2 and this result was confirmed by LAGERKVIST in a preliminary communication (6). The incorporation of isotope in position 4 reported at the same time by this author must be regarded as uncertain considering that the degradation was shown later to be erroneous regarding the separation of C_4 , C_5 and C_6 (paper II).

Recently LAGERKVIST, REICHARD and EHRENSVÄRD (39) have shown that 3- C^{13} , 4- C^{14} -aspartic acid administered to rat liver slices is incorporated into RNA pyrimidines. An interpretation of this finding, however, cannot be given until the distribution of isotope from aspartic acid in the pyrimidine ring has been investigated.

The numerous investigations of purine metabolism have yielded valuable informations especially regarding the precursors of the purine carbon atoms. BUCHANAN

et al. (1) showed that carbon atoms 2 and 8 in uric acid isolated from pigeon excreta were derived from formate, carbon atoms 4 and 5 from the carboxyl and methylene carbons of glycine, respectively, nitrogen 7 from the amino group of glycine (4) and carbon atom 6 from CO₂. These findings have been confirmed for the nucleic acid purines in the rat by ABRAMS *et al.* (5) and HEINRICH and WILSON (7). That formate, glycine and CO₂ are direct precursors of uric acid is furthermore supported by the finding of SCHULMAN *et al.* (40) that pigeon liver extracts incorporate formate, glycine and CO₂ in the molar ratio 2:1:1. KREBS *et al.* (41, 42) were able to demonstrate a piling up of hypoxanthine in pigeon liver slices and it is now generally accepted that this is due to the absence of xanthine oxidase in pigeon liver and that the synthesis of uric acid proceeds via hypoxanthine which is converted to uric acid in the kidneys. GREENBERG (43) in his ingenious investigation of hypoxanthine synthesis has presented strong evidence that phosphoric acid and ribose are attached prior to ring closure to inosinic acid which is then converted to hypoxanthine.

The demonstration by STETTEN *et al.* (44) that a diazotizable substance, later identified as 4-amino-5-imidazole carboxamide (45), is accumulated in *E. coli* during growth inhibition with sulfonamides and the ability of this substance to act as a precursor for purines (46, 47) suggested that the carboxamide might be a normal intermediate in purine synthesis. This possibility has been investigated by several authors but the results obtained so far have not been unequivocal. However, following the work of GREENBERG (43) and SCHULMAN *et al.* (48) it seems reasonable to assume that the carboxamide is not a normal intermediate although it can be converted to an actual intermediate, presumably 4-amino-5-imidazole-carboxamide ribotide, thus accounting for its ability to act as a precursor.

The metabolic origin of the purine nitrogen atoms 1, 3 and 9 is still obscure. Ammonia has long been known to be an able precursor of purine nitrogen (1, 49) but the possibility that ammonia might be metabolically related to any special nitrogen atoms in the purines has not been investigated. Recently SONNE and LIN (50) have reported that the incorporation of isotope from N¹⁵-ammonia into hypoxanthine in pigeon liver extracts is only one fourth of that obtained with N¹⁵-aspartic acid. They conclude that ammonia is not a direct precursor of any of the purine nitrogen atoms. This may well be true if it is interpreted to mean that ammonia as such does not enter the purine ring. However, as can be seen from the results obtained in paper III, ammonia may still be specifically interrelated with some of the purine nitrogen atoms.

Experiments with N¹⁵-Ammonia

In view of the rather high incorporation of isotope from labeled ammonia into uric acid in pigeons (1) and nucleic acid pyrimidines in rats both in vivo (49) and in vitro¹, it was considered possible that the labeling was not due to a random incorporation into all the nitrogen atoms of these substances but that some of them might be specifically derived from ammonia. To test this possibility the following investigations were carried out.

Two experiments with N¹⁵-ammonia on rats were made. The precursor was administered in 4 doses by subcutaneous injection. After 8 hours the animals were sacrificed and the livers and small intestines pooled and homogenized in a Waring blender with alcohol. The polynucleotides were isolated according to HAMMARSTEN

¹ LAGERKVIST, U., and REICHARD, P., unpublished.

(51) and uridine and cytidine from PNA according to REICHARD (8, 38). In one of the experiments the uridine fraction was pooled with the uridine obtained from cytidine by deamination and then degraded as described before. In the other experiment the uridine and cytidine fractions were degraded separately.

Unfortunately the cytidine fraction was lost by accident during the separation of nitrogen atoms 1 and 3 and consequently the distribution of isotope between these atoms in pure cytidine could not be determined. There is, however, little reason to believe that the two pyrimidine ribosides should differ very much from each other in this respect.

The results from these experiments showed that the incorporation of isotope into position 1 was 3.0 times as high as into position 3 when the combined uridine and cytidine fractions were degraded and 3.6 times as high when uridine was degraded separately.

In order to investigate ammonia as a precursor for the uric acid nitrogen atoms two experiments with N^{15} -ammonium chloride on pigeons were made. The birds were kept on a wire screen and were each given 4 subcutaneous injections of the precursor. After 12 hours a first collection of excreta was made and after another 12 hours a second one. Uric acid was isolated according to St. JOHN and JOHNSON (52) as modified by FISHER (53) and then degraded as described above. Two degradations were made in each experiment.

The results of the experiments showed that the isotope was incorporated to about the same extent into positions 3 and 9 and that the incorporation into these positions was much higher than into positions 1 and 7. Only samples from the first 12 hours were degraded since the labeling of uric acid during this period was considerably higher than during the following 12 hours.

Experiments with N^{15} -Aspartic Acid

Considering the fact that the aminogroup of aspartic acid is utilized 4 times as much as ammonia for the synthesis of hypoxanthine in pigeon liver extracts as reported by SONNE and LIN (50) it was thought to be of interest to investigate the distribution of isotope between the nitrogen atoms of uric acid after administration of N^{15} -aspartic acid to pigeons.

Two experiments were made in the same way as described above for N^{15} -ammonium chloride except that only one degradation was made in each experiment. The results showed a comparatively equal distribution of isotope between the uric acid nitrogen atoms in contrast to the findings with ammonia.

Discussion

It is evident from the experiments on rats that nitrogen atom 1 of the ribonucleic acid pyrimidines is in some way metabolically derived from ammonia. Whether or not this is due to an incorporation of ammonia as such into this position of the pyrimidine ring cannot be decided without further investigations. The fixation of CO_2 and ammonia in a derivative of carbamyl-L-glutamate, which has recently been shown to play an important rôle in the synthesis of citrulline from ornithine (54, 55, 56), can be cited as an example of a possible mechanism for the direct incorporation

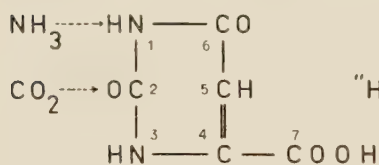
of ammonia into pyrimidines in as much as ammonia and CO_2 enter adjacent positions in the molecule.

On the other hand, ammonia could just as well be incorporated via some intermediate(s) in analogy with the synthesis of arginine from citrulline where the amino group of aspartic acid has been shown to be the immediate precursor (57).

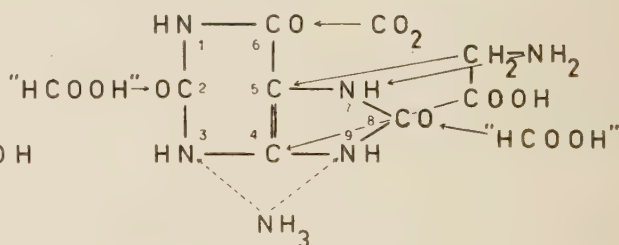
Regarding the experiments with labeled ammonia on pigeons the metabolic origin of nitrogen atoms 3 and 9 of uric acid from ammonia is obvious although the reactions involved are still obscure. There is, however, some evidence to indicate a path via some unknown intermediate(s) as the most plausible explanation, viz. the more extensive utilization of the amino group of aspartic acid compared to ammonia in pigeon liver extracts. The discrepancy between the utilization of ammonia in the intact animal and in liver extracts could then be explained by assuming that the interconversion between ammonia and the hypothetical intermediate(s), although rapid *in vivo*, is in some way impaired in liver extracts.

The results obtained in the experiments with N^{15} -aspartic acid on pigeons do not necessarily mean that the amino group of this amino acid could not be a direct precursor for any of the uric acid nitrogen atoms. It must be kept in mind that the unspecific distribution of isotope from aspartic acid might be due to an impermeability of the liver cells to this amino acid (58). A degradation of labeled hypoxanthine obtained from N^{15} -aspartic acid in pigeon liver homogenate or extract ought to give valuable information regarding this question.

The figures below are intended to visualize our present knowledge of the synthesis of uric acid and ribonucleic acid pyrimidines. Since there is considerable evidence to indicate that orotic acid or a structurally related substance is a normal intermediate in pyrimidine synthesis, this substance has been chosen to represent the pyrimidines. An unbroken arrow represents what is believed to be a direct incorporation of the precursor while a broken arrow means a reaction that may proceed via some intermediate(s).



Orotic acid



Uric acid

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